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TITLE: Chemoprevention of Prostate Cancer Initiation in a Novel Transgenic Mouse Model by Targeting 15-Lipoxygenase-1

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13. SUPPLEMENTARY NOTES

14. ABSTRACT
FLiMP mice, which conditionally express prostatic human 15-LO-1, display mouse prostatic intraepithelial neoplasia (mPIN) by week 20, but do not progress to cancer when on normal diet. To examine for the up-regulated and downregulated genes in the prostates of FLiMP we used pooled cDNA sets of individual prostate regions from FLiMP$^{+/-}$ and FLiMP$^{+/+}$ mice and age matched nontransgenic C57BL/6 littermates were used for cDNA production. Hybridization was performed on pretreated 38.5K mouse Illumina MEEBO oligonucleotide (25,000 genes) microarray slides. The slides were scanned and intensity data were further analyzed with GeneSpring 7.0 software and normalized. All of the data were filtered very heavily yielding a total of 7037 genes. This list was used as the starting list for fold-change calculations (p< 0.001, fold change > 2). Fold change calculations were performed and genes were pooled and uploaded into the PANTHER gene expression analysis tool (http://panther.appliedbiosystems.com) to examine for pathways. The levels of Phospholipase C-gamma 2 (PLC-γ2) mRNA increased with age and were 8 times higher at week 32 and that a similar pattern in the levels of Phosphatase and tensin homolog (PTEN) and SMARCA3 related Switch/Sucrose non-fermentable (SWI/SNF) mRNA decreased with age, both of which were 4 times lower at week 32 in all prostate lobes of the FLiMP mice. Consequence of such differences in the expression levels of these genes that have wide roles in the process of prostate carcinogenesis in relation to the susceptibility of mouse prostate glands to PIN via 15-LO-1 expression is currently unknown. We will exploit the utility of these markers to examine the n-6 and n-3 fatty acids effects on PIN development.

15. SUBJECT TERMS
Diet, polyunsaturated fatty acids (PUFAs), 15-lipoxygenase-1, cyclooxygenase, prostate cancer, Array, Genes.

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INTRODUCTION:

Fifteen lipoxygenase-1 (15-LO-1) in the Mouse Prostate (FLiMP)- A novel mouse model to study impact of omega diets on prostate cancer progression: To gain better mechanistic insight of the role of 15-LO-1 in prostate cancer (PCa), we recently developed a novel C57BL/6 transgenic mouse called FLiMP [1]. These mice, which conditionally express prostatic human 15-LO-1, display mouse prostatic intraepithelial neoplasia (mPIN) by week 20, but do not progress to cancer when on normal diet. Therefore, FLiMP mice provide an excellent model to study the experimental paradigm of PCa initiation, highlighting their usefulness in evaluating early "proactive" intervention strategies in PCa. Our proposed studies are predicated on the hypothesis that dietary prevention is an effective means of eradicating PCa, and that FLiMP mice provide a valuable pre-clinical model for chemoprevention studies. Diets rich in either omega (ω)-3 or ω-6 polyunsaturated fatty acids (PUFAs) directly impact PCa tumor growth. Furthermore, the FLiMP mice, which overexpress human 15-LO-1, faithfully recapitulate the early stages of human PCa progression. These observations support the potential value of ω-3 fatty acid SDA as a chemopreventative agent and the need for further studies. Thus, the FLiMP mouse model has the strength of being a genetically defined, immune-competent tool to address the ω-6 and ω-3 experimental paradigm.

During the past years we have already bred the transgenic FLiMP several times. In order to avoid potential genetic drift in FLiMP mice and variations in expected outcomes, we have begun re-deriving the FLiMP mice. We have already started a few cohorts and still awaiting for sufficient male mice to finish Task 2. However, we still believe that it will take longer than 36 months to accomplish our Aims successfully. We have conserved our budget for this study accordingly and requesting an additional 1 year no-cost extension to successfully complete the studies.

Task 2. **Characterize key molecular events altered in prostate cancer initiation in FLiMP$^{+/+}$ mouse model by dietary n-6 and n-3 (Months 8-36):**

a. Sacrifice batches/groups of experimental (43 mice fed with n-6 diet, 31 mice fed with n-3 diet and 10 mice fed with normal diet) and control wt mice (10 mice fed with each of the diets) at 5 time points (week 8, 11, 14, 24 and 36), dissect dorsolateral (test) and anterior (control) prostate lobes, aliquot tissues for RNA extraction and quantitative PCR, immunohistochemistry, phospholipid analysis, high performance liquid chromatography (HPLC) and mass spectrometry analyses for 15-lipoxygenase-1 (15-LO-1) and cyclooxygenase-(COX)-2 enzyme activities and metabolites (Months 8-18).
b. Perform immunohistochemistry analyses of the prostates and quantitate proliferation, apoptosis, human 15-LO-1 and endogenous mouse 12/15-LO [control] (Months 9-24).
c. Annual report will be written (Month 24).
d. Fatty acid composition analyses from red blood cells and from prostate at one final time point i.e., immediately after sacrifice to examine for uniform dietary phospholipid levels in both tissues and validate that fatty acids in red blood cell membranes as an accurate estimate of the distribution of fatty acids in the diet as well as prostate (Months 9-28).
e. HPLC and mass spectrometry analyses for 15-LO-1 and COX-2 enzyme activities and metabolites to examine for the effect of n-3 on n-6 pathways (Months 28-32)
f. Quantitative RT-PCR and Western blot analyses of PLC-γ, PTEN and SMARCA-3 marker genes to monitor changes in expression from dorsolateral (test) and

anterior (control) prostate lobes of mice from each diet group and at 5 time points (Months 30-35).

g. Data compilation, statistical analyses, report preparation, annual report and publication/s (Months 9-36).

BODY:

PRELIMINARY data:

Complementary [c]-DNA target preparation and microarray hybridization:

Pooled sets of individual prostate regions from FLiMP$^{+/-}$ and FLiMP$^{+/+}$ mice and age matched nontransgenic C57BL/6 littermates were used for cDNA production. Three microarrays were hybridized for each age and strain (with the same pools cDNA) using a common reference for all hybridizations that consisted of cDNA from age matched nontransgenic C57BL/6 littermates. For each hybridization, amplified sample RNA was labeled with Cy5 and amplified control RNA was labeled with Cy3 in a reverse transcriptase reaction. Hybridization was performed on pretreated 38.5K mouse Illumina MEEBO oligonucleotide (25,000 genes) microarray slides (MI Microarrays Inc., Nashville, TN). The slides were scanned with the GenePix scanner 4000B and associated software (Axon Instruments, Union City, CA). Scanned intensity data were further analyzed with GeneSpring 7.0 software (Silicone Genetics, Redwood City, CA) and normalized by Lowess. The Lowess (also termed as locally weighted scatter plot smoothing procedure) normalization approach is a durable set of base routines, written in Fortran in the late 1970s and now widely used in microarray analysis. Lowess cleans the data as a function of one predictor for data with normal errors or for data with long-tailed symmetric errors (robust fitting). However, because gene expression data contains only a single predictor variable, this approach is basically interchangeable and provides robust normalization of microarray data through a polynomial regression approach.

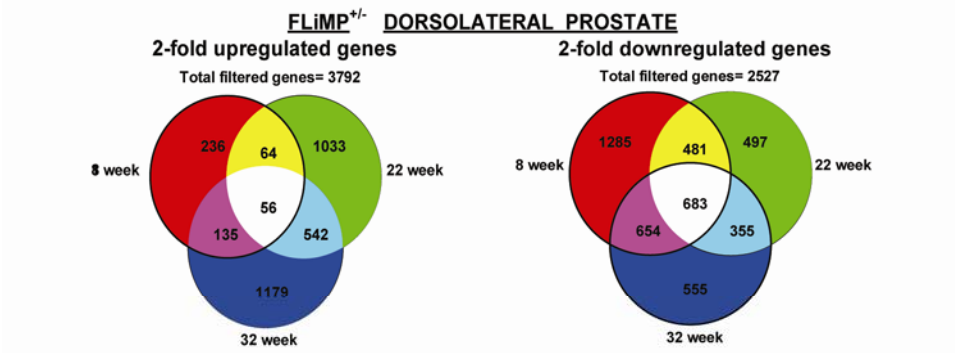

Figure 1: Venn diagram of all the genes in the dissected dorsolateral prostate lobe (DLP) of FLiMP$^{+/-}$ mice (Reprentative, n=3) that showed at least 2-fold up (A) or 2-fold down regulation (B) at 8, 22 and 32 weeks of age. Gene data was filtered to eliminate unreliable data points such as flagged spots (by the initial, image-extracting software), lowly expressed spots, and spots with negative signal values. Each circle contains the total number of genes that were regulated in that sample. Common genes are viewed in the areas intersecting the circles. For example, the white area represents common genes regulated at all ages. The common genes from white area were analyzed for both FLiMP$^{+/-}$ and FLiMP$^{+/-}$ using the PANTHER gene expression analysis tool to identify genes from potential pathways.

All of the data were filtered very heavily to eliminate all data points that may not be reliable. This included flagged spots (by the initial, image-extracting software), lowly expressed spots, and spots with negative signal values yielding a total of 7037 genes. This list was used as the starting list for fold-change calculations (p< 0.001, fold change > 2). Fold change calculations were performed by filtering the above list to examine for changes of at least 2-fold up- or down-regulation in each of the hybridizations. Also of interest were genes with a unique pattern of expression that did not change in the 8 week time period, but showed increased expression over week 22, and further over-expression at week 32 (late-response genes). Likewise, genes that showed increased expression in the 10-week period (early response genes), but showed increased down-regulation over weeks 21 and 35 were also of interest. Also of interest, were the common genes that showed differential expression in all prostate lobes of the FLiMP$^{+/+}$ and FLiMP$^{+/-}$ mice when compared within the same age group (**Figure 1**). These genes

were pooled and uploaded into the PANTHER gene expression analysis tool (http://panther.appliedbiosystems.com) to examine for pathways.

Resulting pathways are shown in **Figure 2A, 2B, 2C and 2D**. It is intriguing that the levels of Phospholipase C-gamma 2 (PLC-γ2) mRNA increased with age and were 8 times higher at week 32 and that a similar pattern in the levels of Phosphatase and tensin homolog (PTEN) and SMARCA3 related Switch/Sucrose non-fermentable (SWI/SNF) mRNA decreased with age, both of which were 4 times lower at week 32 in all prostate lobes of the FLiMP mice. Consequence of such differences in the expression levels of these genes that have wide roles in the process of prostate carcinogenesis in relation to the susceptibility of mouse prostate glands to PIN via 15-LO-1 expression is currently unknown. We will exploit the utility of these markers to examine the n-6 and n-3 fatty acids effects on PIN development. It is clear, however, that the role of these genes in the development of PIN and possibly to prostate cancer deserves rigorous investigation. We are still in process of rederiving FLiMP mice and obtain required numbers to perform the proposed experiments.

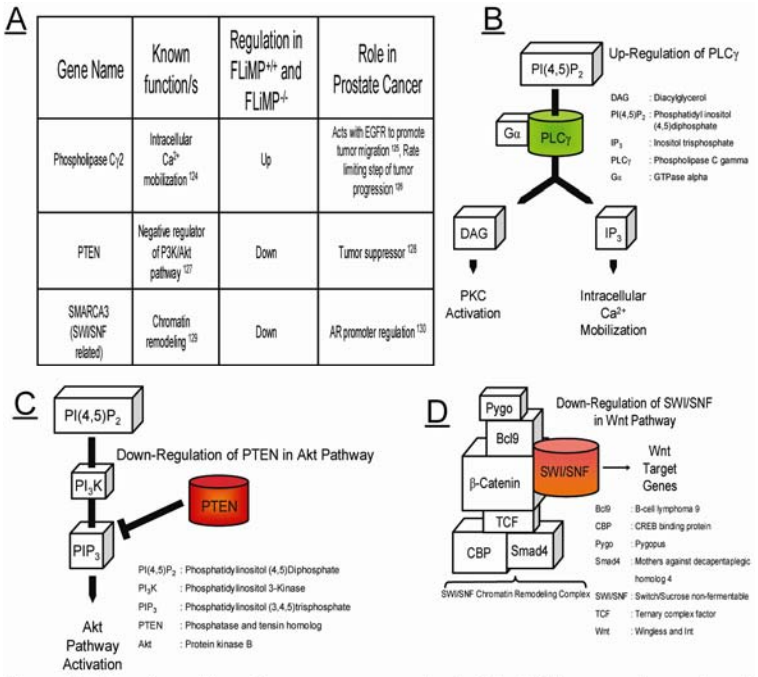

Figure 2: Mapping of baseline gene expression [>2-fold difference (up or down) with age] represented in all prostate lobes of FLiMP$^{+/+}$ and FLiMP$^{+/-}$ mice.

KEY RESEARCH ACCOMPLISHMENTS IN YEARS 1 and 2:

(1) Wild type mice did not exhibit any prostate-specific phenotypic changes regardless of their diet.

(2) Given that FLiMP$^{+/+}$ mice express 15-LO-1 and that the these enzymes convert n-6 LA to the pro-tumorigenic metabolite, 13-HODE, as expected, FLiMP$^{+/+}$ mice fed a diet high LA diet exhibited more aggressive PIN, with PIN-like changes observed in as early as 8-10 weeks compared to FLiMP$^{+/+}$ mice fed a normal diet (PIN observed) and,

(3) Our study in year 2 provided mechanistic roles of omega (ω)-3 fatty acids in slowing PCa growth by altering ω-6/ ω-3 ratios via diet, and promoting apoptosis and inhibiting proliferation in tumors by directly competing with ω-6 fatty acids for 15-LO-1, and COX-2 activities.

REPORTABLE OUTCOMES: None.

CONCLUSION: Omega (ω)-3 fatty acids can slow PCa growth by altering ω-6/ ω-3 ratios via diet, and promote apoptosis and inhibit proliferation in tumors by directly competing with ω-6 fatty acids for 15-LO-1, and COX-2 enzyme activities.

REFERENCES: List all references pertinent to the report using a standard journal format (i.e. format used in *Science, Military Medicine*, etc.).

1. Kelavkar, U.P., Parwani, A.V., Shappell, S.B. and Martin, W.D. (2006) Conditional expression of human 15-lipoxygenase-1 in mouse prostate induces prostatic intraepithelial neoplasia: the FLiMP mouse model. *Neoplasia*, **8**, 510–522.

APPENDICES: None.